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Steric Hindrance Mutagenesis versus Alanine Scan in Mapping of Ligand Binding Sites in the Tachykinin NK₁ Receptor

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ABSTRACT

Residues in transmembrane domain (TM)-III, TM-V, TM-VI, and TM-VII believed to be facing the deep part of the presumed main ligand-binding pocket of the NK₁ receptor were probed by alanine substitution and introduction of residues with larger and/or chemically distinct side chains. Unaltered or even improved binding affinity for four peptide agonists, substance P, substance P-O-methyl ester, cladoisin, and neurokinin A, as well as normal EC₅₀ values for substance P in stimulating phosphatidylinositol turnover indicated that these mutations did not alter the overall functional integrity of the receptor. The alanine substitutions in general had only minor effects on nonpeptide antagonist binding. However, the introduction of the larger and polar aspartic acid and histidine residues at positions corresponding to the monoamine binding aspartic acid in TM-III of the β_2 -adrenoceptor (ProIII:08, Pro112 in the NK₁ receptor) and to the presumed monoamine interacting "two serines" in TM-V (ThrV:09, Thr201; and IleV:12, Ile204) impaired by >100-fold the binding of a group of nonpeptide antagonists, including CP96,345, CP99,994, RP67,580, RPR100,893, and CAM4092. In contrast, another group of nonpeptide antago-

nists, LY303,870, FK888, and SR140,333, were little or not at all affected by the space-filling substitutions. Two of these compounds, FK888 and LY303,870, were those most seriously affected (75–89-fold) by alanine substitution of PheVI:20 located in the upper part of the main ligand-binding crevice. Surprisingly, substitution of AlaIII:11 (Ala115), which is located in the middle of TM-III, conceivably pointing toward TM-VII, with a larger valine residue increased the affinity for all 13 ligands tested, presumably by creating a closer interhelical packing. It is concluded that the introduction of larger side chains at positions at which molecular models indicate that this is structurally allowed can be a powerful method of locating ligand-binding sites due to the considerable difference between positive and negative results. Such steric hindrance mutagenesis strongly indicates that one population of nonpeptide antagonists bind in the deep pocket of the main ligand-binding crevice of the NK₁ receptor, whereas another group of nonpeptide antagonists, especially SR140,333, was surprisingly resistant to mutational mapping in this pocket.

In G protein-coupled receptors with 7TM, a main ligand-binding crevice is believed to be located between the loops and the outer segments of TM-III, TM-IV, TM-V, TM-VI, and TM-VII (Schwartz and Rosenkilde, 1996). The adrenoceptors were among the first 7TM receptors to be cloned and subsequently characterized in respect of ligand-binding sites. Through a multidisciplinary effort using receptor mutagenesis, affinity cross-linking, and biophysical approaches with fluorescent probes, strong evidence was gathered in favor of the notion that monoamine agonists bind to a pocket located deep in the transmembrane domain of the receptor structure among TM-III, TM-IV, TM-V, and TM-VI (Strader *et al.*, 1994). For example, in the β_2 -adrenoceptor, norepinephrine

is presumed to bind through its amine function to a conserved aspartic acid residue in TM-III (AspIII:08) and to make an aromatic-aromatic interaction with a phenylalanine residue in TM-VI as the catechol ring of the ligand is oriented by hydrogen bond formation between its hydroxyl groups and two serine residues located one helical turn apart in TM-V (Strader *et al.*, 1994) (Fig. 1). Only recently was the stereospecific recognition of the β -OH group of the agonist isoproterenol shown to occur through interaction with an asparagine residue located one helical turn more exterior in TM-VI, AsnVI:20 (Wieland *et al.*, 1996). Mutational mapping of binding sites for other monoamines indicates that they presumably also bind to this deep pocket in the main ligand-binding crevice of their respective receptors through interaction with the corresponding or neighboring residues as identified in the β_2 -adrenoceptor (Schwartz, 1994; Strader *et al.*, 1994; Strange, 1996; Strosberg, 1993).

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ABBREVIATIONS: 7TM, seven transmembrane segments; BH, Bolton-Hunter; PCR, polymerase chain reaction; TM, transmembrane domain; SP-OMe, substance P-O-methyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

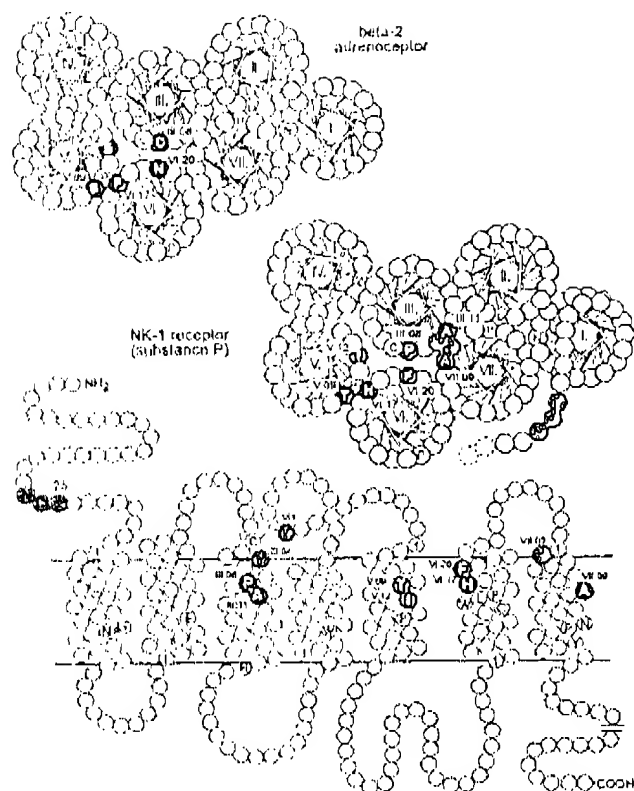


Fig. 1. Helical wheel diagram of the β_2 -adrenoceptor and helical wheel and serpentine diagram of the tachykinin NK₁ receptor. The helical wheel diagrams are built over the rhodopsin structure (Scherf et al., 1993) as interpreted by Baldwin (1993) with TM-III as the central helix and in the counter-clockwise orientation as viewed from outside the cell, which recently was probed experimentally (Elling and Schwartz, 1996). Helix/helix interaction has been optimized on the basis of metal ion site engineering (Elling et al., 1995; Elling and Schwartz, 1996). White letters on black, residues presumed to be involved in agonist binding in the β_2 -adrenoceptor; black letters on gray, residues previously shown to be involved in substance P binding in the NK₁ receptor (Fong et al., 1992; Huang et al., 1994; Kage et al., 1996). In the NK₁ receptor, residues probed by alanine substitution and steric hindrance mutagenesis in the current study are shown (white letters on black). Generic numbering system for residues in 7TM receptors indicates helix number (roman type) followed by the residue number (italic type separated by a colon) here and in the text (Baldwin, 1993).

Among 7TM receptors, one of the most thoroughly studied peptide systems is the tachykinin NK₁ receptor. In contrast to monoamine receptors but in analogy with other peptide receptors, several presumed interaction points for the endogenous ligand substance P have been mapped to the amino-terminal extension and to extracellular loops, as well as to the most exterior helical turn of, for example, TM-III and TM-VII (Fig. 1) (Fong et al., 1992; Huang et al., 1994). Extensive mutational analysis performed by several groups has, however, failed to identify an interaction point for substance P in the actual transmembrane domain of the NK₁ receptor corresponding to the site at which monoamines are presumed to bind and activate their receptor (Fong et al., 1992, 1993, 1994a, 1994b; Gether et al., 1993b, 1994a, 1994b; Huang et al., 1994; Strader et al., 1994; Zoffmann et al., 1993) [for a discussion of the effect of substitutions on the hydrophilic, presumed inner face of TM-II, see Huang et al. (1994) and

Rosenkilde et al. (1994)]. However, this deep portion of the main ligand-binding crevice is where mutations have indicated that nonpeptide antagonists are binding. Importantly, the identification of many of the presumed interaction points for nonpeptide antagonists has in fact been based on rather small effects on binding affinity. For example, it is generally presumed by others (Fong et al., 1994a; Zoffmann et al., 1993) as well as by the current investigators that the prototype nonpeptide antagonist CP96,345 is interacting with HisVI:17 (His265); however, alanine substitution of HisVI:17 in fact hardly affects the binding of CP96,345. The presumed interaction of CP96,345 with this residue is based on the effect of mutations on analogs of CP96,345, not the compound itself, which obviously is a much weaker augmentation (Fong et al., 1994a; Zoffmann et al., 1993).

Mutational analysis of binding sites in 7TM receptors as well as most other proteins has been performed through alanine scan mutagenesis (Schwartz, 1994). This procedure, in which the side chain at a particular position basically is just "removed," is in general relatively safe in terms of not disturbing the overall structure of the protein. However, alanine substitutions may not be very effective at locating contact residues in binding sites. Based on the X-ray structure of human growth hormone in complex with its receptor and from the exhaustive mutational analysis of both of these proteins performed by Clackson and Wells (1995), it seems that a surprisingly small fraction of the actual contact residues in the ligand/receptor complex are really important for the binding energy. Alanine substitution of most of the residues in the interface between the hormone and its receptor did in fact not impair significantly the binding affinity (Clackson and Wells, 1995). In the case of 7TM receptors, it could be argued that a peptide such as substance P may obtain most of its binding energy through interactions with residues in the exterior part of the receptor, but it could still activate the receptor through interactions with residues corresponding to those to which monoamines bind. If so, alanine substitutions in the transmembrane segments of the NK₁ receptor would have given false-negative results.

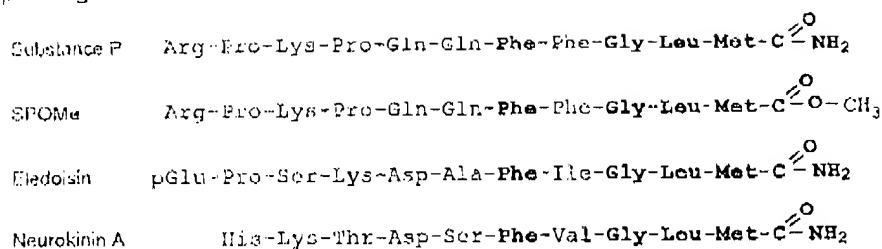
In the current study, the deep part of the main ligand-binding crevice, as defined by the binding site for isoproterenol in the β_2 -adrenoceptor, is probed in the NK₁ receptor by both alanine substitution and steric-hindrance mutagenesis (i.e., substitutions with larger side chains, which are assumed to fill up the presumed pocket). The introduction of larger side chains was performed at positions at which our molecular models of the receptor indicated that such substitutions would be "allowed" (i.e., where they would not be expected to harm helix/helix interactions). These molecular models have been optimized by, for example, the construction of a series of interhelical metal ion sites (Elling et al., 1995; Elling and Schwartz, 1996). Four structurally related peptide agonists and nine nonpeptide antagonists, most of which are structurally distinct, were tested in these NK₁ constructs.

Experimental Procedures

Materials. All peptides were purchased from Peninsula (St. Helens, Merseyside, UK). Nonpeptide compounds for which the structures are shown in Fig. 2 were kindly donated. CP96,345 and CP99,994 were provided by Dr. John A. Lowe III (Pfizer, Groton, CT) (Snider et al., 1991; Rosen et al., 1993). CGP49,823 was provided by

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Peptide agonist



Non-peptide antagonist

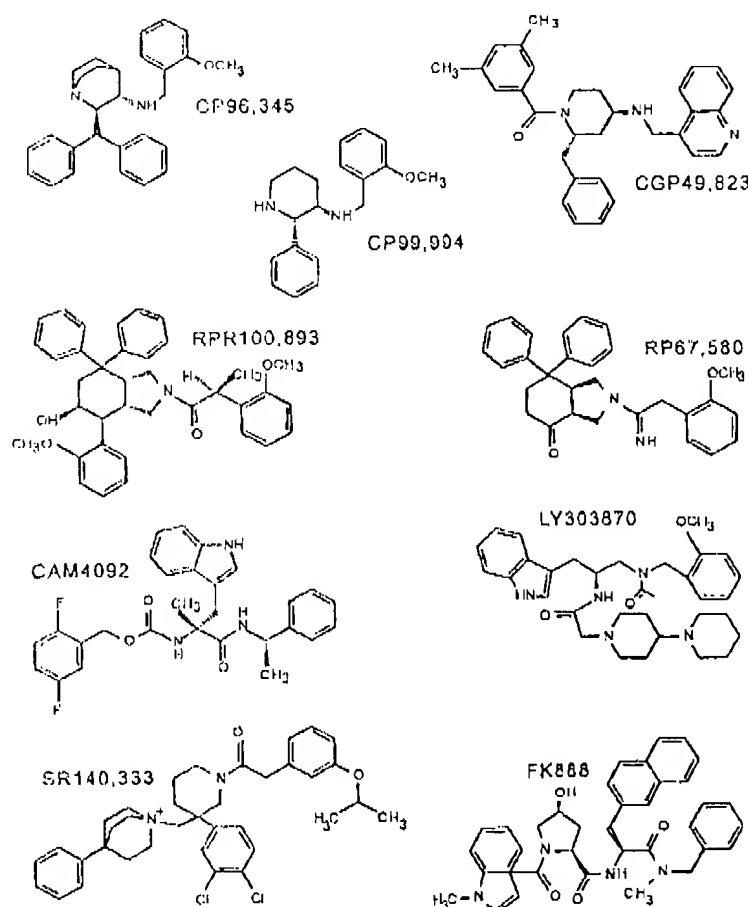


Fig. 2. Structures of peptide agonists and nonpeptide antagonists for the NK₁ receptor used in the current study. **Bold**, four amino acid residues common to all four tachykinin peptides. The lead compounds for the nonpeptide antagonists CP96,345 (Snider *et al.*, 1991), CP99,994 (Rosen *et al.*, 1993), RP67,580 (Garret *et al.*, 1991), RPR100,893 (Tabert and Peyronel, 1994), LY303,870 (Citter *et al.*, 1995), and SR140,333 (Edmonds-Alt *et al.*, 1993b) were found by screening of chemical files, whereas the initial lead compounds for CGP49,823 (Schilling *et al.*, 1993), CAM4092 (Boyle *et al.*, 1994), and FK888 (Fujii *et al.*, 1992) were peptide-based compounds.

Dr. Walter Shilling (Ciba/Novartis, Basel, Switzerland) (Schilling *et al.*, 1993). RP67,580 and RPR100,893 were provided by Dr. Claude Garret (Rhône-Poulenc, Paris, France) (Garret *et al.*, 1991; Tabert and Peyronel, 1994). CAM4092 was provided by David Howell (Parke-Davis, Cambridge, UK) (Boyle *et al.*, 1994). LY303,870 was provided by Dr. Philip Hipskins (Eli Lilly, Indianapolis, IN) (Citter *et al.*, 1995). SR140,333 was provided by Drs. Xavier Edmonds-Alt and Jean-Claude Brelière (Sanofi Recherche, Montpellier, France) (Edmonds-Alt *et al.*, 1993b). FK888 was provided by Drs. T. Fujii and D. Hagiwara (Fujisawa, Osaka, Japan) (Fujii *et al.*, 1992). *Pfu* polymerase was from Stratagene (La Jolla, CA). AG 1-X8 anion-exchange resin was from BioRad Laboratories (Hercules, CA). *myo*-[³H]inositol (1 Ci/mmol), BII reagent (specific activity, 2000 Ci/mmol), and

Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP were from Amersham (Little Chalfont, UK).

Construction of mutant receptors. The cDNA encoding the wild-type human NK₁ receptor was cloned into the eukaryotic expression vector pTEJ-8 (Johansen *et al.*, 1990). Mutations were constructed by PCR using either (1) the overlap extension method (Horton *et al.*, 1989) or (2) a combined extension of mutated internal primers by T4 DNA polymerase followed by selective amplification of the mutated DNA strand by PCR (Stappert *et al.*, 1992). The PCR products were digested with appropriate restriction endonucleases, purified, and cloned into the pTEJ8-NK₁. All PCR experiments were performed using *pfu* polymerase according to the instruction of the manufacturer. All mutations were verified by restriction endonucle-

ase mapping and subsequent DNA sequence analysis using the Thermo Sequase fluorescent labeled primer cycle sequencing kit with 7-deaza dGTP on an ABI-express DNA sequencer according to the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden).

Transfections and tissue culture. COS-7 cells were grown in Dulbecco's Modified Eagle's Medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. The expression plasmids containing the cDNAs encoding the wild-type or mutant receptors were transiently expressed after transfection according to the calcium phosphate precipitation method (Gether *et al.*, 1993a).

Binding experiments. Monoiodinated [¹²⁵I]-BII-labeled substance P was prepared and purified by high performance liquid chromatography (Gether *et al.*, 1993a). Transfected COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells per well was determined by the apparent expression efficiency of the individual clones with an aim of 5–10% binding of the added radioligand. Two days after transfection, cells were assayed by competition binding for 3 hr at 4° using 25 pM [¹²⁵I]-BII-labeled substance P plus varying amounts of unlabeled ligand in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, supplemented with 150 mM NaCl, 5 mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 40 µg/ml bacitracin. Nonspecific binding was determined as the binding in the presence of 1 µM substance P. Determinations were made in triplicate.

Phosphatidylinositol assay. One day after transfection COS-7 cells (0.3×10^6 cells/well) were incubated for 24 hr with 5 µCi of *myo* [³H]inositol in 1 ml of inositol-free Dulbecco's 1885 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin per well. Cells were washed twice in PI buffer consisting of 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CoCl₂, 10 mM glucose, and 0.05% (w/v) bovine serum albumin and were incubated in 0.5 ml of PI buffer supplemented with 10 mM LiCl at 37° for 30 min. After stimulation with increasing concentration of substance P for 45 min at 37°, cells were extracted with 10% ice-cold perchloric acid followed by incubation on ice for 30 min. The resulting supernatant was neutralized with KOH in HEPES buffer, and the generated [³H]inositol phosphates were purified on BioRad AG 1-X8 anion-exchange resin (Berridge *et al.*, 1983). Determinations were made in triplicate.

Calculations. IC₅₀ and EC₅₀ values were determined by nonlinear regression, and K_i and B_{max} values were calculated using the Inplot 4.0 software (GraphPAD Software, San Diego, CA).

Results

In the NK₁ receptor, residues located in the presumed deep part of the main ligand-binding crevice at positions corresponding to the interaction points for agonists in the β₂-adrenoceptor were substituted with alanine or with amino acid residues presenting larger, polar side chains.

Substitutions of ProIII:08 (Pro112). The important, conserved aspartic acid in TM-III of monoamine receptors, which most convincingly has been implicated in binding of the amine function through complementary chemical modifications performed on the ligand and receptor (Strader *et al.*, 1991), is a proline residue in the NK₁ receptor, ProIII:08 (Pro112). This residue was substituted by alanine, aspartic acid, and histidine, of which the latter two would be expected to occupy considerably more space than the pyrrolidine ring of the proline. Furthermore, these two residues would change the local environment considerably by introducing polarity and possibly charge in front of TM-III in this part of the main ligand-binding pocket (Fig. 1). Because ProIII:08 (Pro112) does not correspond to one of the highly conserved proline residues of the transmembrane helices in 7TM receptors, it

was not expected that substitution with a "normal" amino acid residue in general would perturb the receptor structure by altering the backbone configuration at this site. This assumption was confirmed by the normal agonist binding and signal transduction observed in these constructs (see below).

Alanine substitution of ProIII:08 (Pro112) did not impair the binding of substance P or any of the other peptide agonists, as determined in competition binding experiments using radiolabeled substance P in whole-cell binding experiments performed in transiently transfected COS-7 cells (Table 1). In fact, the binding of one of the peptide agonists, SPOMe, was improved 20-fold (F_{mut} = 0.04). Of the nine nonpeptide antagonists studied, only the binding of CP99,994 was clearly impaired by the proline for alanine substitution, and the impairment was only 13-fold (Table 1). In contrast, introduction of an aspartic acid residue at this position decreased the binding affinity of seven of the nine nonpeptide antagonist by >10-fold, and five of the compounds were decreased by >100-fold (Fig. 3, Table 1). Substitution with a histidine residue had a similar, detrimental effect on the binding of the nonpeptide compounds, as observed with the aspartic acid residue at this position leading to a 180–4600-fold reduction in affinity for five of the compounds (Fig. 3, Table 1). Although the binding of most of the antagonists was affected seriously, a single nonpeptide antagonist, LY303,870, was not affected, and two other antagonists, SR140,333 and FK888, were only slightly affected by aspartic acid and histidine substitutions of ProIII:08 (Fig. 3, Table 1).

Substitution of ThrV:09 (Thr201) and IleV:12 (Ile204). In TM-V of the β₂-adrenoceptor serine residues located at two positions one helical turn apart, SerV:09 (Ser204) and SerV:12 (Ser207) are believed to be involved in the formation of hydrogen bonds to the hydroxyl groups of the catechol ring (Strader *et al.*, 1994). In the NK₁ receptor, these positions are occupied by a threonine residue, ThrV:09 (Thr201), and an isoleucine residue, IleV:12 (Ile204), respectively (Fig. 1). ThrV:09 was mutated to alanine, isoleucine, or histidine, whereas IleV:12 was probed by only histidine substitution and only in combination with a similar exchange at position V:09. Similar results were obtained by either "removing" the polar side chain of ThrV:09, as in the alanine substitution, or by introducing the slightly larger and apolar side chain of isoleucine at this position (Table 2). Among all the ligands tested, the binding of only two nonpeptide antagonists, CAM4092 and LY303,870, was impaired by these two mutations (Table 2). In fact, the binding of three of the peptide agonists was improved 2–4-fold (Table 2). In contrast, introduction of a histidine residue at position V:09 relatively selectively reduced the binding affinity of the two structurally related nonpeptide antagonists, CP96,345 and CP99,994 (Table 2). Interestingly, residue V:09 is located in an *i* + 4 position [i.e., one helical turn] below HisV:05 (His197), which previously has been demonstrated to be a crucial contact point for CP96,345 (Fong *et al.*, 1993; Gether *et al.*, 1993b, 1994b).

The combined introduction of histidine residues at both position V:09 and V:12 impaired the binding of eight of the nine nonpeptide antagonists; only the FK888 compound was unaffected (Table 2). The quinuclidine prototype antagonist CP96,345 was most seriously affected (with an 850-fold reduction in affinity) in this double mutation in which both

TABLE 1
Binding affinities of peptide agonists and nonpeptide antagonists in the wild-type human NK₁ receptor and in mutant forms with substitutions in TM-III and -VII. Peptides were expressed transiently in COS-7 cells, and competition binding experiments were performed as described in the text using [³⁵S]-labeled substance P. B_{max} values for the various constructs in fold¹⁰⁰ cells are: hNK1 wild-type, 1.6 ± 0.5 ; (P12D), 6.5 ± 0.6 ; (P12H), 7.5 ± 3.2 ; (A12V), 5.9 ± 1.6 ; and (A294V), 5.4 ± 1.5 .

	hNK1 wild-type		(P12D) (H108)		(P12H) (H108)		(A12V) (H108)		(A294V) (V109)	
	K_i	K_d	K_i	K_d	K_i	K_d	K_i	K_d	K_i	K_d
Substance P	0.11 ± 0.04 (12)	0.15 ± 0.03 (14)	1.6	0.07 ± 0.01 (7)	0.6	0.07 ± 0.04 (9)	0.05 ± 0.01 (12)	0.3	0.20 ± 0.05 (10)	2.6
SPOMe	6.3 ± 1.4 (7)	9.20 ± 0.95 (3)	0.00	4.8 ± 0.3 (2)	0.8	6.5 ± 2.2 (4)	0.41 ± 0.19 (5)	0.07	2.9 ± 0.7 (3)	0.5
Leu	21 ± 7 (9)	62 ± 38 (4)	3.0	19 ± 2 (4)	0.9	19 ± 5 (5)	5.8 ± 2.6 (6)	0.3	60 ± 16 (4)	2.9
Neurokinin A	14 ± 3 (9)	40 ± 13 (5)	2.9	16 ± 3 (4)	1.1	14 ± 4 (5)	4.3 ± 1.1 (5)	0.3	38 ± 3.2 (3)	2.7
CP96345	0.31 ± 0.05 (6)	1.1 ± 0.1 (9)	3.5	45 ± 5 (13)	150	65 ± 8 (4)	0.14 ± 0.05 (3)	0.5	0.61 ± 0.05 (4)	2.0
CP99994	0.28 ± 0.05 (9)	3.5 ± 0.3 (4)	13	1203 ± 300 (5)	4300	1300 ± 200 (2)	0.10 ± 0.01 (3)	0.4	1.1 ± 0.2 (4)	3.9
RP67580	19 ± 4 (7)	36 ± 1 (5)	1.9	270 ± 60 (8)	14	3400 ± 800 (4)	3.8 ± 0.4 (6)	0.2	480 ± 130 (6)	25
RP100283	19 ± 3 (9)	35 ± 8 (5)	1.8	>10000 (5)	>530	>10000 (4)	3.4 ± 0.2 (4)	0.2	38 ± 4 (5)	2.0
CAM4092	3.5 ± 0.8 (8)	15 ± 2 (4)	4.3	1300 ± 600 (6)	370	1300 ± 400 (4)	0.72 ± 0.18 (5)	0.2	11 ± 1 (3)	3.1
CGP49823	0.69 ± 0.13 (8)	2.9 ± 0.2 (3)	4.2	54 ± 16 (5)	78	4.9 ± 0.9 (4)	0.26 ± 0.05 (4)	0.4	4.9 ± 0.8 (4)	7.1
LY303870	0.38 ± 0.08 (7)	0.37 ± 0.04 (3)	1.0	0.60 ± 0.14 (4)	1.6	0.49 ± 0.15 (4)	0.05 ± 0.01 (5)	0.2	0.62 ± 0.08 (3)	1.6
F3688	1.1 ± 0.1 (7)	1.4 ± 0.4 (4)	1.3	7.8 ± 1.8 (5)	7.1	3.1 ± 0.4 (4)	0.38 ± 0.09 (4)	0.3	1.6 ± 0.5 (3)	1.5
SR140333	0.44 ± 0.10 (7)	0.87 ± 0.31 (4)	2.0	2.7 ± 0.8 (5)	6.1	5.1 ± 0.9 (4)	0.07 ± 0.02 (5)	0.2	0.59 ± 0.50 (3)	1.3

* P_{mut} is calculated as K_i mutant receptor/ K_i (wild-type hNK1 receptor). This corresponds to fold decrease in affinity. Values are mean \pm standard error.

substitutions are located below HisV:05 (His197). Importantly, the binding of neither substance P nor of any of the other peptide agonists was impaired by this space-filling, double mutation in the deep part of the main ligand-binding pocket of the NK₁ receptor; in fact, the affinity for three of the tachykinin peptides was improved 3–4-fold (Table 2).

Substitutions of PheVI:20 (Phe268). In TM-VI, two residues have been implicated in agonist binding in the β_2 -adrenoceptor: PheVI:17 and AsnVI:20. The major interaction site for the catechol ring itself, PheVI:17 (Phe290) is in the NK₁ receptor already occupied by a residue with a relatively large side chain [i.e., the histidine residue HisVI:17 (His265)] (Fig. 1). This position has been probed extensively with multiple different substitutions, including phenylalanine, and in no case was any effect on substance P binding observed, whereas the binding of nonpeptide antagonists of several classes was impaired by these mutations (Pong *et al.*, 1994a; Zoffmann *et al.*, 1993). Therefore, we did not further address HisVI:17.

The β -OH group of the agonist isoproterenol interacts with AsnVI:20 (Asn293) in the β_2 -adrenoceptor (Wieland *et al.*, 1996). VI:20 is an interesting position because it is located in the outer segment of TM-VI pointing more-or-less directly into the main ligand-binding pocket toward TM-III and it is the most superficial or exteriorly located of the presumed interaction points for monoamine ligands (Fig. 1). The relative position of residue VI:20 in the 7TM receptor structure is rather well established as based on metal ion binding sites constructed between residue VI:24 located one helical turn above VI:20 and two positions in TM-V, V:01 and V:05 (Elling *et al.*, 1995). Alanine substitution of the corresponding PheVI:20 in the NK₁ receptor impaired the binding of all of the nonpeptide antagonists between 5- and 89-fold (Table 3). Interestingly, in this case, the binding of the PK888 and LY303,870 antagonists, which were resistant to substitutions located more deeply in the pocket, was affected most seriously, 75- and 89-fold, respectively. However, SR140,333 was relatively unaffected even by this substitution (Table 3). Substance P binding was impaired 11-fold, but the three other tachykinin peptides were basically unaffected by the PheVI:20-to-alanine substitution (Table 3). Substitution with either tryptophan or histidine at this position had little or no effect on the binding affinity of any of the many different ligands except for a few scattered hits of low magnitude (Table 3). In fact, all the peptide ligands bound better to the tryptophan-substituted receptor mutant than to the wild-type receptor (Table 3). Thus, although it cannot be determined whether the impairing effect of the alanine substitution is directly or indirectly mediated, the "removal of the side chain" in this case had a much more pronounced effect than substitution with the more polar and, for tryptophan, much larger side chain.

Steric hindrance mutagenesis of alanine residues in the transmembrane segments. The occurrence of an alanine residue on the inner face of a transmembrane helix will conceivably create a hole in the protein structure due to the small size of the side chain. This hole can then be occupied by a side chain from another, interdigitating helix, or it can function as part of the binding site for a receptor ligand. Two such alanine residues were probed by steric hindrance mutagenesis through substitution with valine (i.e., introduction of two extra methyl groups).

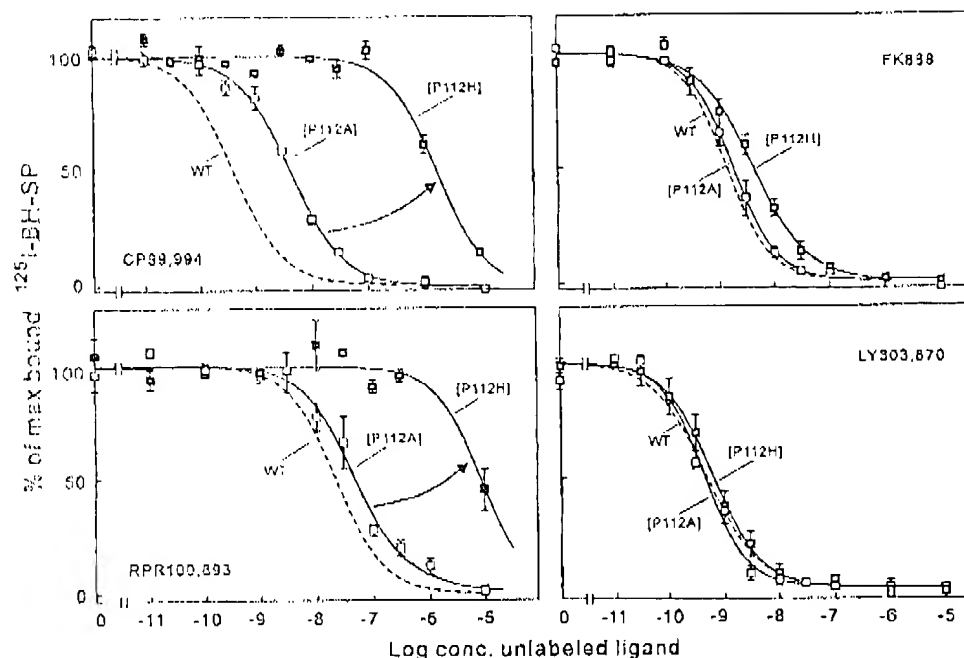


Fig. 3. Competition binding curves for four nonpeptide compounds in the wild type (WT) NK₁ receptor and in two mutant forms with substitution of Pro112:08 (Pro112) that corresponds to the main monoamine interaction point in TM-III, Asp111:08 (Asp113). CP99,994 and RPR100,893 represent the group of compounds that are seriously affected by the steric hindrance mutations, and FK838 and LY303,870 represent compounds that are less or not at all affected (see tables). Binding experiments were performed on whole-cell binding on transiently transfected COS-7 cells using ¹²⁵I-BH-labeled substance P as radioligand.

TABLE 2

Binding affinities of peptide agonists and nonpeptide antagonists in the wild-type human NK₁ receptor and in mutant forms with substitutions in TM-V.

Receptors were expressed transiently in COS-7 cells, and competition binding experiments were performed as described in the text using ¹²⁵I-BH-labeled substance P. B_{max} values for the various constructs in COS-7 cells were hNK1 wild-type, 15 ± 5 ; (T201A), 130 ± 22 ; (T201H), 30 ± 9 ; (T201H), 20 ± 8 ; and (T201H; T204H), 6.0 ± 1.1 .

	hNK1 wild-type	(T201A) (V:09)	Fmut ^a	(T201H) (V:09)	Fmut	(T201H) (V:09)	Fmut	(T201H; T204H) (V:09; V:12)	Fmut
	K_i	K_i		K_i		K_i		K_i	
	nM (n)			nM (n)		nM (n)		nM (n)	
Substance P	0.11 ± 0.01 (12)	0.19 ± 0.03 (12)	1.7	0.14 ± 0.03 (3)	1.3	0.23 ± 0.05 (4)	2.1	0.28 ± 0.02 (13)	2.6
SPOMe	6.3 ± 1.4 (7)	3.4 ± 0.2 (2)	0.5	1.5 ± 0.1 (3)		9.0 ± 1.2 (3)	1.4	1.1 ± 0.3 (5)	0.2
Fla	21 ± 7 (9)	8.3 ± 4.7 (3)	0.4	9.3 ± 2.0 (3)	0.4	44 ± 3 (3)	2.1	5.4 ± 1.0 (5)	0.3
Neurokinin A	14 ± 3 (9)	6.8 ± 2.9 (3)	0.5	4.6 ± 0.4 (3)	0.3	23 ± 6 (3)	1.6	3.9 ± 0.9 (6)	0.3
CP96,345	0.31 ± 0.05 (6)	0.40 ± 0.04 (9)	1.3	0.79 ± 0.16 (6)	2.5	14 ± 3 (3)	45	260 ± 60 (5)	850
CP99,994	0.28 ± 0.05 (9)	0.49 ± 0.11 (3)	1.8	0.36 ± 0.04 (3)	1.3	3.2 ± 0.2 (3)	11	21 ± 4 (4)	75
RP67,580	19 ± 4 (7)	17 ± 3 (4)	0.9	26 ± 2 (3)	1.4	120 ± 10 (3)	6.3	370 ± 70 (3)	20
RPR100,893	19 ± 3 (9)	13 ± 3 (3)	0.7	10 ± 1 (3)	0.5	130 ± 10 (3)	0.8	2000 ± 680 (8)	110
CAM4092	3.5 ± 0.8 (9)	150 ± 40 (5)	43	120 ± 10 (3)	34	11 ± 1 (3)	3.1	33 ± 7 (4)	9.4
CG149,420	0.69 ± 0.13 (3)	0.69 ± 0.31 (3)	1.0	0.49 ± 0.07 (4)	0.7	1.5 ± 0.5 (4)	2.2	11 ± 1 (4)	16
LY303,870	0.38 ± 0.05 (7)	26 ± 11 (4)	68	4.6 ± 0.8 (3)	12	3.9 ± 1.1 (4)	10	3.6 ± 0.9 (4)	10
FK838	1.1 ± 0.1 (7)	3.0 ± 1.4 (4)	2.7	1.1 ± 0.3 (4)	1.0	1.5 ± 0.3 (3)	1.4	1.0 ± 0.4 (4)	0.9
SH149,333	0.44 ± 0.10 (7)	0.40 ± 0.10 (5)	0.9	0.33 ± 0.08 (3)	0.8	0.93 ± 0.17 (3)	2.1	4.2 ± 1.6 (4)	9.5

^a Fmut is calculated as K_i (mutant receptor)/ K_i (wild-type hNK1 receptor). This corresponds to fold decrease in affinity. Values are mean \pm standard error.

AlaIII:11 (Ala115) is located one helical turn below ProII:08 (Pro112) in TM-III, presumably facing toward TM-II and TM-VII (Fig. 1). The introduction of a valine residue at this position did not impair the binding of any of the ligands; on the contrary, the affinity of all 13 ligands was increased an average of 5-fold (Table 1). Thus, the space-filling substitution in this particular case seemed to be beneficial for the binding of both agonists and antagonists. The compound that was most affected was SPOMe, with an increase in affinity of ~20-fold (Table 1).

AlaVII:09 (Ala294) is located a couple of helical turns deep in TM-VII and is presumably facing toward TM-VI and TM-III (Fig. 1). Valine substitution of AlaVII:09 impaired the binding of only one of the nonpeptide antagonist, RP67,580 (26 fold). This compound also is affected by other substitu-

tions in the interface among TM-III, TM-VI, and TM-VII (Huang *et al.*, 1994; Schwartz TW, unpublished observations). All other ligands displayed either unaltered or slightly reduced affinity in the ValVII:09 mutant form of the NK₁ receptor (Table 1).

Signal transduction experiments. The results presented show that neither alanine substitutions nor steric hindrance mutations in the deep part of the main ligand-binding pocket affected the affinity of peptide agonists on the NK₁ receptor as determined in ligand-binding experiments. Nevertheless, theoretically the peptides could obtain most of their binding energy through interactions with residues located in the more exterior parts of the receptor but still perform the actual activation of the receptor through interaction with residues located deep in the transmembrane do-

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TABLE 3

Binding affinities of peptide agonists and nonpeptide antagonists in the wild-type human NK₁ receptor and in mutant forms with substitutions in position VI:20 (Ile268)

Receptors were expressed transiently in COS-7 cells, and competition binding experiments were performed as described in the text using ¹²⁵I-BH-labeled substance P. B_{max} values for the various constructs in fmoI/10⁵ cells were hNK1 wild-type, 15 ± 5; [F268A], 24 ± 4; [F268W], 4.7 ± 1.0; and [F268H], 12 ± 3.

	hNK1 wild-type	[F268A] (VI:20)		[F268W] (VI:20)		[F268H] (VI:20)	
	K _i	K _i	F _{mut} ^a	K _i	F _{mut} ^a	K _i	F _{mut} ^a
	nM (n)	nM (n)		nM (n)		nM (n)	
Substance P	0.11 ± 0.04 (12)	1.2 ± 0.1 (17)	11	0.025 ± 0.003 (4)	0.2	0.52 ± 0.08 (6)	4.7
SPOMe	0.3 ± 1.4 (7)	12 ± 4 (3)	1.9	5.0 ± 0.8 (3)	0.8	3.4 ± 1.2 (3)	0.5
Rel	21 ± 7 (9)	22 ± 3 (3)	1.0	9.8 ± 1.5 (3)	0.5	27 ± 12 (3)	1.3
Neurokinin A	14 ± 3 (9)	15 ± 1 (3)	1.1	5.3 ± 0.4 (3)	0.4	20 ± 9 (3)	1.4
CP96,345	0.31 ± 0.05 (6)	22 ± 2 (10)	71	0.43 ± 0.09 (3)	1.4	1.1 ± 0.2 (5)	3.5
CP99,994	0.28 ± 0.05 (9)	4.3 ± 0.7 (4)	15	0.19 ± 0.02 (3)	0.7	0.85 ± 0.21 (8)	3.0
[P67,580]	19 ± 4 (7)	700 ± 50 (3)	37	11 ± 4 (3)	0.6	59 ± 3 (8)	3.1
RPR100,893	19 ± 3 (9)	100 ± 30 (3)	5.3	31 ± 1 (3)	1.6	54 ± 7 (3)	2.8
QAM1092	3.5 ± 0.8 (9)	100 ± 10 (3)	29	10 ± 3 (4)	2.9	17 ± 1 (2)	4.9
GGP49,823	0.69 ± 0.13 (8)	24 ± 4 (3)	35	12 ± 2 (4)	17	5.3 ± 0.3 (3)	7.7
LY303,870	0.38 ± 0.08 (7)	34 ± 8 (5)	89	1.2 ± 0.2 (3)	3.2	1.4 ± 0.5 (3)	3.7
PF588	1.1 ± 0.1 (7)	82 ± 3 (3)	75	1.9 ± 0.1 (3)	1.7	6.5 ± 1.2 (3)	5.9
SR140,337	0.41 ± 0.10 (7)	2.1 ± 0.1 (3)	4.8	0.69 ± 0.20 (3)	1.6	1.6 ± 0.4 (3)	3.6

^a F_{mut} is calculated as K_i (mutant receptor)/K_i (wild-type hNK1 receptor). This corresponds to fold decrease in affinity.

main. However, as shown in Fig. 4 and in Table 4, substance P was able to stimulate phosphatidylinositol turnover in all these mutant receptors with a similar EC₅₀ value as observed in the wild-type NK₁ receptor. In the mutant receptors, the E_{max} value for the substance P-induced phosphatidylinositol turnover was also similar and the same as that of the wild-type receptor, except for the construct with the double histidine substitution in TM-V and the alanine and histidine substitution of PheVI:20, which demonstrated a 3–5-fold reduction in maximal response to substance P (Table 4).

Discussion

One of the major problems in mutational mapping of binding sites is the difficulty in ruling out false-negative results (Schwartz *et al.*, 1995). This is demonstrated, for example, in the case of growth hormone and its receptor, for which the actual binding site now is known due to X-ray analysis of the receptor/ligand complex (Clackson and Wells, 1995). False-negative results are mainly a problem when alanine substitutions are used because this procedure basically creates only an extra "hole" in the presumed binding pocket through

TABLE 4

Ability of substance P to stimulate phosphatidylinositol turnover in the wild-type human NK₁ receptor and in mutant forms of this expressed as EC₅₀ and E_{max} values

Receptors were expressed transiently in COS-7 cells, and phosphatidylinositol turnover experiments with dose-response curves for substance P were performed as described in the text and as shown for the wild-type NK₁ receptor and the [P112D] and [P112H] mutant forms of this in Fig. 4.

	EC ₅₀	E _{max}
	nM	fmoI/10 ⁵ cells (n)
hNK1 wild-type	0.42 ± 0.08	38 ± 6 (3)
[P112A] (III:08)	0.50 ± 0.18	44 ± 11 (3)
[P112D] (III:08)	0.32 ± 0.03	67 ± 43 (3)
[P112H] (III:08)	0.56 ± 0.11	32 ± 4 (3)
[A115V] (III:11)	0.82 ± 0.08	49 ± 17 (3)
[T201A] (V:09)	0.50 ± 0.21	70 ± 9 (2)
[T201I] (V:09)	0.67 ± 0.01	67 ± 4 (2)
[T201H] (V:09)	1.1 ± 0.21	68 ± 12 (2)
[T201H, I204H]	2.1 ± 0.7	7 ± 2 (3)
[F268A] (VI:20)	2.1 ± 0.5	13 ± 4 (4)
[F268W] (VI:20)	0.51 ± 0.12	26 ± 16 (3)
[F268H] (VI:20)	2.2 ± 0.8	8 ± 1 (3)
[A294V] (VII:09)	1.9 ± 0.3	44 ± 19 (5)

Values are mean ± standard error.

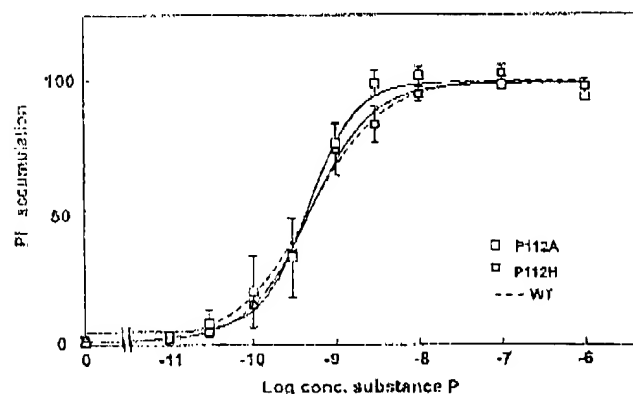


Fig. 4. Dose-response curves for stimulation of phosphatidylinositol (PI) turnover in response to substance P in the wild-type (WT) NK₁ receptor and in mutant forms of this with substitution of Pro112:08 (Pro112) to aspartic acid and histidine, respectively.

removal of the side chain. The effect of an alanine substitution is highly dependent on the relative contribution to the binding energy of the replaced residue. A residue may be part of the ligand/receptor interface without in fact contributing much to the total binding energy (Clackson and Wells, 1995). However, the introduction of a larger side chain in a presumed binding pocket conceivably would cause many more problems for the ligand by impairing the interaction not only with the mutated residue but presumably also with neighboring residues due to the incongruence that would be created in a larger part of the interface. In the current study, this is reflected in the observed 100–1000-fold decrease in affinity for a number of nonpeptide antagonists as the result of the introduction of larger and polar residues, such as aspartic acid and histidine, in the deep part of the main ligand-binding crevice between TM-III and TM-V in the NK₁ receptor (Tables 1 and 2). It is on the basis of these significant, positive "hits" that the lack of effect of such substitutions on the binding of substance P and related tachykinin

peptide agonists as well as a group of nonpeptide antagonists is viewed as strong evidence in favor of the notion that the peptides and certain nonpeptides do not use this deep pocket as part of their binding site in the NK₁ receptor.

Implications for nonpeptide antagonist binding. The steric hindrance mutants in the deep pocket among TM-III, TM-V, and TM-VI result in a very substantial loss of affinity for a number of the nonpeptide antagonists acting on the NK₁ receptor (Tables 1 and 2). These results combined with the results of a number of previous studies performed with some of these compounds suggest strongly that the binding sites for compounds such as CP96,345, CP99,994, RP67,580, RPR100,893, and CAM1092 are in fact located in this pocket (Schwartz, 1994; Schwartz *et al.*, 1995; Strader *et al.*, 1994). For compounds such as CGP49,823 and LY303,870, the results point in the same direction, but the effects of the steric hindrance mutations were not as consistent. FK888 was resistant to all kinds of mutational mapping performed in the deep pocket, but together with LY303,870, it was the ligand that was most seriously affected by the alanine substitution at position VI:20 located at the exterior border of the pocket, indicating that these compounds may be binding mainly in the more shallow part of the main ligand-binding crevice (Fig. 6). It should be noted that false-positive results are still difficult to rule out. Thus, it is impossible to differentiate between the effect of substituting an actual contact residue between the receptor and a ligand and the effect of substituting a second row residue (Schwartz *et al.*, 1995). However, the fact that the peptide agonists could bind normally and activate signal transduction in these constructs indicates that the overall structure of the receptor was not perturbed.

One nonpeptide antagonist, SR140,333, was either unaffected or only slightly affected by all the mutations of the current study. On the basis of the dramatic effect of the steric hindrance substitutions in the deep pocket on several other compounds, it seems likely that SR140,333 does not interact with this part of the receptor. Because SR140,333 is in fact not seriously affected by any other point mutation yet performed in the NK₁ receptor (Schwartz TW, unpublished observations), it could be speculated that either it binds to a part of the receptor that has not yet been probed by mutagenesis (which is hardly possible if it is on the outside of the receptor) or its binding energy is a result of multiple relatively weak interaction points (it is a relatively large compound) (Fig. 1). A third, interesting hypothesis is that SR140,333 is able to exploit several receptor conformations with almost equal affinity. In that case, the compound would escape mutational mapping if the side chain that is mutated was not of major importance in both receptor conformations. This would mean that several point mutations must be combined to seriously affect the binding of such compounds. Interestingly, small chemical modifications of SR140,333 create compounds that are high affinity, selective NK₂ or NK₃ ligands (Emonds-Alt *et al.*, 1993a). For the homologous nonpeptide antagonist SR48,968, it has been reported that the combination of a couple of point substitutions is required to affect the binding of this compound, which is very similar structurally to the NK₂ receptor (Huang *et al.*, 1995).

Implications for peptide agonist binding. As shown in the current study, it is possible to fill out the deep pocket of the main ligand-binding crevice of the NK₁ receptor without affecting the binding or function of substance P or the bind-

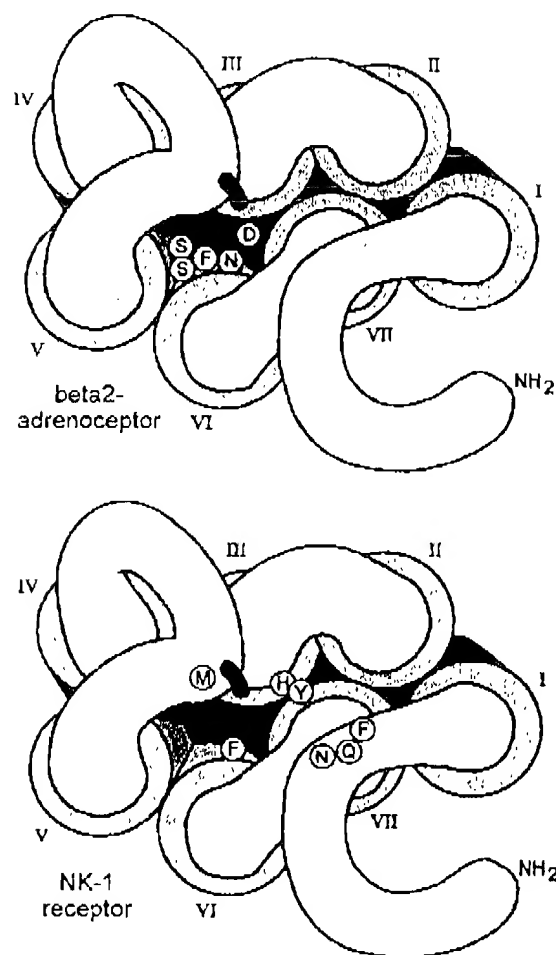


Fig. 6. Outside-in views of the main ligand-binding crevice of the β_2 -adrenoceptor and substance P NK₁ receptor with presumed contact residues for agonists (black letters on white). In the NK₁ receptor, these residues have been identified through receptor mutagenesis in the amino-terminal segment of TM-III (His110, His108) (Fong *et al.*, 1992) and TM-VII (Tyr287, Tyr287) (Huang *et al.*, 1994) and by affinity cross-linking in exterior loop 2 (Met181) using photoreactive, radiolabeled substance P (Kage *et al.*, 1996). Steric hindrance mutagenesis in the current study indicates that many nonpeptide antagonists bind in the deep pocket corresponding to the presumed binding site for monoamines in their receptors but that this is not the case for the peptide agonists and, importantly, that several nonpeptide antagonists, such as FK888, LY303,870, and SR140,333, also do not map to this pocket. PheVI:20 (Phe268), which corresponds to the presumed interaction point for the β -OH group of isoproterenol in the β_2 -adrenoceptor, AsnVI:20 (Asn293), has been implicated as a possible contact point for both substance P and a number of nonpeptide antagonists in the current study (see Table 3).

ing and function of three other high affinity peptide agonists of this receptor. Of the five main presumed interaction points for agonists in the β_2 -adrenoceptor, only one, position VI:20 (which is the most exteriorly located site) seems to be shared by substance P in the NK₁ receptor. Previously, site-directed mutagenesis and affinity cross-linking have been used to identify presumed contact points for substance P in the amino-terminal exterior segment of the receptor, in extracellular loop 2 close to the disulfide bridge connecting the top of TM-III, and at the most exterior parts of TM-III and TM-VII

(i.e., in the more "shallow" part of the main ligand-binding crevice) (Pang et al., 1992; Huang et al., 1994; Kage et al., 1996; Li et al., 1995) (Figs. 1 and 5). The substitution of a number of residues down along the inner, hydrophilic face of TM II in the NK₁ receptor does impair the ability of substance P to compete with radiolabeled antagonists seriously (Huang et al., 1994). However, the affinity of the peptide as measured in homologous binding assays and bioassays is hardly affected (Huang et al., 1994; Rosenkilde et al., 1994). Thus, it is unlikely that these deeply located residues are part of the high affinity binding site for substance P (Rosenkilde et al., 1994).

The current results argue against the presence of a "common lock" for all agonist "keys" in 7TM receptors located in the deep pocket among TM-III, TM-V, and TM-VI because this is where monoamines but apparently not substance P binds (Schwartz and Rosenkilde, 1996). Another important piece of evidence against the existence of such a common trigger area in this deep pocket is the observation that 7TM receptors, including monoamine receptors, can be activated by antibodies reacting with extracellular loops or even raised against synthetic peptides corresponding to the extracellular loops (Schwartz and Rosenkilde, 1996). Importantly, the currently preferred models for receptor activation are all variations over a two-state model, in which the crucial element is the ability of the receptor to induce intracellular signaling by itself (Kenakin, 1995; Lefl, 1995; Leskowitz et al., 1993; Schwartz et al., 1995). In a system in which the decisive change from inactive to active conformation can occur automatically (i.e., independent of agonist binding), there is no need for an induced intramolecular signaling event to be precipitated by ligand interaction with a particular active site, or "common lock" (Schwartz and Rosenkilde, 1996).

Recently, it was demonstrated by electron paramagnetic spectroscopy performed on rhodopsin after introduction of a series of pairs of spin labels that receptor activation is associated with a major conformational change. This was interpreted as being a relative movement and rotation of TM-VI away from TM-III (Farrens et al., 1996). Blockade of rhodopsin function through engineering of disulfide bridges or interhelical metal ion sites between TM-III and TM-VI is in agreement with this notion (Farrens et al., 1996; Sheikh et al., 1996). Interestingly, the only apparent overlap in presumed agonist binding sites in the β_2 -adrenoceptor and NK₁ receptor, position VI:20, is located at the interface between TM-III and TM-VI (Fig. 5). Thus, it could be argued that in both cases, the agonists act by binding between TM-III and TM-VI and through this interaction can induce, or more likely stabilize, the correct, active conformation of their respective receptors. The monoamines seem to achieve this by binding in the deep part of the main ligand-binding crevice; the larger and more polar peptide ligand, substance P, achieves this by binding in the more shallow part of this crevice (Figs. 1 and 5). Thus, although there may not be an actual common binding site or trigger area that agonists must touch to activate 7TM receptors, as discussed above, there could be a common receptor conformation or set of conformations that all agonists must be able to stabilize or induce for the G proteins to recognize the receptor as being active (Schwartz et al., 1995). The question of stabilization versus induction can perhaps be considered more of a seman-

tic than an actual distinction depending on the degree of constitutive activity in the receptor system (Kenakin, 1996).

Does PheVI:20 (Phe268) represent a common interaction point for peptide agonists and nonpeptide antagonists? It has been surprisingly difficult to locate common interaction points in the NK₁ receptor for substance P and competitive nonpeptide antagonists, although a relatively large number of interaction sites have been identified for each ligand (Gelber et al., 1993b; Schwartz, 1994; Schwartz et al., 1995; Strader et al., 1994). PheVI:20 seems to be the first good candidate for such a residue, although the effect on peptide affinity is only 11-fold and the binding of the homologous peptides was not affected (Table 3). It cannot be excluded that the generally negative effect on ligand binding of substitution of the naturally occurring, large phenylalanine residue with the smaller alanine residue could be a result of an indirect effect. The facts that substitution of PheVI:20 with the equally large or even larger side chains of tryptophan and histidine had almost negligible effects on the binding of all 13 tested ligands and that most compounds were negatively affected by the alanine substitution at this position could be taken as support for the notion that the effect of the alanine substitution is indirect. However, it could equally well be argued that both tryptophan and histidine could substitute well for the natural phenylalanine residue in a direct receptor/ligand interaction. In the overall picture of presumed interaction points for substance P, PheVI:20 fits rather well between the residues at the outer segments of TM-III and TM-VII and Met181 located next to the disulfide bridge from the top of TM-III, which by affinity cross-linking has been implicated as a major interaction site for substance P (Kage et al., 1996) (Figs. 1 and 5). Thus, it seems likely that PheVI:20 could form part of the "inner wall" or "floor" of the binding site for substance P in the outer portion of the main ligand-binding crevice of the NK₁ receptor. If the effect of alanine substitution of PheVI:20 on the binding of the nonpeptide antagonists is also considered to be a direct effect, then this residue is the first example of the otherwise surprisingly elusive common interaction point for substance P and nonpeptide antagonists. However, as previously discussed, it is a matter of opinion whether such a single residue should be considered to represent a case of overlap in binding site in a common rigid mold or instead forms part of different binding sites occurring in different receptor conformations (Schwartz et al., 1995). We favor the latter possibility, although the answer to this question will not be known until the actual three-dimensional structures of the various receptor/ligand complexes have been determined.

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COPY

Initials: HEW/RHU/PGA/smc Docket No.: 2225.1001-009 Date: 9/11/00

This is to acknowledge receipt of the following documents each filed under Certificate of Mailing Procedure 37 C.F.R. 1.8:

- ☒ Transmittal of Proposed Drawing Corrections and New Formal Drawings w/ copy and Copies of Figures 1 & 2 with changes noted in red
- ☒ DRAWINGS Sheets 1/4-4/4
- ☒ Other: Second Preliminary Amendment with Fee Transmittal w/ copy

Applicants: Loetscher and Moser

Application No.: 09/607,156

Filed: June 29, 2000

Title: IP-10/MIG RECEPTOR DESIGNATED CXCR3, NUCLEIC ACIDS, AND METHODS OF USE THEREFOR

Date received by the PTO:



OPMA\HOFMANN\Manager\274480.1
HEW/RIU/saj
December 4, 2001

PATENT APPLICATION
DOCKET NO. 2225.1001-009

Expedited Procedure under 37 C.F.R. 1.116
Examining Group 1646

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Marcel Loetscher and Bernhard Moser

Application No.: 09/607,156

Group: 1646

Filed: June 29, 2000

Examiner: J. Murphy

For: IP-10/MIG RECEPTOR DESIGNATED CXCR3, NUCLEIC ACIDS, AND
METHODS OF USE THEREFOR

CERTIFICATE OF FACSIMILE TRANSMISSION	
I hereby certify that this correspondence is being facsimile transmitted to the United States Patent and Trademark Office:	
on <u>12-4-01</u>	<u>Sandra Jammal</u>
Date	Signature
<u>Sandra Jammal</u>	
Typed or printed name of person signing certificate	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is Amendment After Final Rejection Under 37 C.F.R. 1.116 for filing in the above-identified application.

☐ Small entity status of this application under 37 C.F.R. 1.9 and 1.27 has been established by a Small Entity Statement previously submitted.

☐ A Small Entity Statement to establish small entity status under 37 C.F.R. 1.9 and 1.27 is enclosed.

The fee has been calculated as shown below:

(COL. 1)	(COL. 2)	(COL. 3)
	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NO. PREVIOUSLY PAID FOR
TOTAL	30	MINUS * 30
INDEP	8	MINUS ** 8
FIRST PRESENTATION OF MULTIPLE DEP. CLAIM		

* not fewer than 20

** not fewer than 3

SMALL ENTITY	
RATE	ADDIT. FEE
X \$9	\$
X \$42	\$
+ \$140	\$

TOTAL = \$ 0

OTHER THAN SMALL ENTITY	
RATE	ADDIT. FEE
X \$16	\$ 0
X \$84	\$ 0
+ \$280	\$ 0

TOTAL = \$ 0

09/607,156

-2-

Please charge Deposit Account No. 08-0380 for the following fees:

<input checked="" type="checkbox"/> [X]	Petition for two month Extension of Time	\$	<u>400</u>
<input type="checkbox"/> []	Amendment Fee	\$	<u> </u>
<input type="checkbox"/> []	Other Fees:		
		\$	<u> </u>
		\$	<u> </u>
	TOTAL:	\$	400

A check is enclosed in payment of the following fees:

<input type="checkbox"/>	Petition for [] month Extension of Time	\$ _____
<input type="checkbox"/>	Amendment Fee	\$ _____
<input type="checkbox"/>	Other Fees:	
	_____	\$ _____
	_____	\$ _____
	TOTAL:	\$ 0

☒ A general authorization is hereby granted to charge Deposit Account No. 08-0380 for any fees required under 37 C.F.R. 1.16 and 1.17 in order to maintain pendency of this application.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

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Dated: December 4, 2001